BIOFABRICATION AND EXTRACTION OF SILVER NANOPARTICLES USING LEAF EXTRACT OF *Azadirachta indica*

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science In Life Science

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"I dedicate my dissertation work to my beloved parents and my adorable brothers. Special thanks to my best friend, Nahid Naushad."

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CERTIFICATE

*This is to certify that the thesis entitled "**Biofabrication and extraction of silver nanoparticles using leaf extract of Azadirachta indica**" submitted by **Ms. Safiya Sultana** (Roll No: 412LS2062) in partial fulfilment of the requirements for the award of **Master of Science in Life Science** to the National Institute of Technology, Rourkela, is an authentic and original record of research work carried out by her under my supervision and guidance.*

To the best of my knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

11/05/14

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DECLARATION

I,Safiya Sultana hereby declare that, this project report entitled **"Biofabrication and extraction of Silver Nanoparticles using leaf extract of *Azadirachta indica*"**, submitted by me, under the guidance of Dr. Suman Jha, Assistant Professor, N.I.T., Rourkela is my own and has not been submitted to any other University or Institute or published earlier.

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Date: 11.5.14. Safiya Sultana

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ABSTRACT

Development of reliable, economical and eco-friendly process for the synthesis of silver nanoparticles is a major step in the field of nanotechnology. There are various conventional approaches involving chemical methods, which are associated with environmental toxicity. Green synthesis approach has emerged as an alternative method to the conventional approach, as it is more economical, biocompatible and environmentally benign. Monodisperse nanoparticles of size less than 100 nm are currently in demand for wide range of applications in different fields of industry. One of the steps to achieve this objective is to use natural reducing agents. This study manifests the synthesis of small sizedand stable silver nanoparticles using the plant extract of *Azadirachta indica*.For detailed study of the morphology and structure of fabricated silver nanoparticles, characterization was done using UV-Vis,ATR-FTIR,DLS,ZETA and SEM. UV–visible spectrum of the aqueous solution containing silver nanoparticles showed a peak centred at 430 nm, and this is due to surface plasmon resonance of silver nanoparticles. SEM image exhibited the formation of well-dispersed and spherical shaped silver nanoparticle in the range of 50–100 nm.ATR-FTIR analysis confirmed the presence of significant amount of reducing entities.It further revealed that phytochemicals like flavanoids and terpenoids present in Neem leaf extract were the main entities to stabilize the synthesized nanoparticles. The synthesized silver nanoparticles were isolated from aqueous solution containing silver nanoparticles using size exclusion chromatography (Sephadex G100). The purified silver nanoparticles were found to be more stable for longer period.

Keywords :Biofabrication,nanosilver,green synthesis

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CONTENTS

Sl. No	LIST OF FIGURES	PAGE NO.
Figure 1.	Image showing several applications of nanoparticles	**12**
Figure 2.	Various approaches for the synthesis of nanoparticles .	**16**
Figure 3.	The top-down approach versus the bottom-up approach.	**18**
Figure 4.	Plant mediated synthesis of nanosilver.	**18**
Figure 5	*Pics of Azadirachta indica leaves.*	**24**
Figure 6	A: 1mM AgNO3 without plant extracts . B: 1mM AgNO3 with Neem extract after 18 hours	**30**
Figure 7	Shows the absorption spectrum biosynthesized silver nanoparticles from Neem plant extract of 5 ratio 30:1,60:1,120:1,240:1 and 3.3:1.	**30**
Figure 8	UV- visible spectra of *Azadirachta indica* as a function of time in different incubations with silver nitrate (1m M) after 4h, 5h and 18 h respectively. The peak 430nm corresponds to the plasmon resonance of silver nanoparticles	**30**
Figure 9	DLS result for 3.3:1 ratio silver nanoparticles.	**31**
Figure 10	Zeta analysis result for3.3:1 ratio silver nanoparticles.	**32**
Figure 11	SEM image for 3.3:1 ratio silver nanoparticles.	**33**
Figure 12	XRD result for 3.3:1 ratio silver nanoparticles.	**34**
Figure13	FTIR result for 3.3:1 ratio silver nanoparticles.	**35**
Figure 14	Purification of Nanoparticle using size exclusion chromatography	**36**
Figure 15	UV- visible spectra of different Eluentsof 3.3:1 ratio silver nanoparticles.	**37**
Figure 16	DLS & Zeta of eluent 1	**38**
Figure 17	DLS & Zeta of eluent 2	**39**
Figure 18	DLS & Zeta of eleunt 3	**39**
Figure.19	DLS& Zeta of eleunt 4	**39**

LIST OF TABLE

List of abbreviation

SEC	Size exclusion chromatography
UV-Vis	Ultraviolet - Visible Spectroscopy
FE-SEM	Field Emission Scanning Electron Microscope
DLS	Dynamic Light Scattering
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infra-Red Spectroscope
XRD	X-Ray Diffraction
SDS	Sodium dodecyl sulphate
AgNP	Silver nanoparticles
nm	Nanometer
Min	Minute
mM	Milimolar
mV	Millivolt
rpm	Rotation per minute

INTRODUCTION

Nanotechnology is a latest field of modern research dealing with synthesis, design and manipulation of nanosized particles. Remarkable development in nanotechnology has opened a novel and very wide frontier of application, that includes the synthesis of nanoscale material, exploration of their unique physical chemical and optoelectronic properties. Nanotechnology has gained tremendous importance in state of the art techniques for health care, food technology , cosmetics, environmental health, mechanics, optics, , chemical industries, biomedical, electronics, space engineering, energy science, drug-gene delivery, optoelectronics, catalysis, light emitters , nonlinear optical devices, single electron transistors, and photo electrochemical applications etc [1-4]. Currently, silver nanotechnology, is becoming popular due to its above extensive applications and distinctive properties (*e.g.* size and shape dependent several properties such as, optical, magnetic and electrical properties), which can be incorporated into biosensor materials, antimicrobial applications, composite fibres, cosmetic products, cryogenic superconducting materials, and electronic components **[figure 1**].

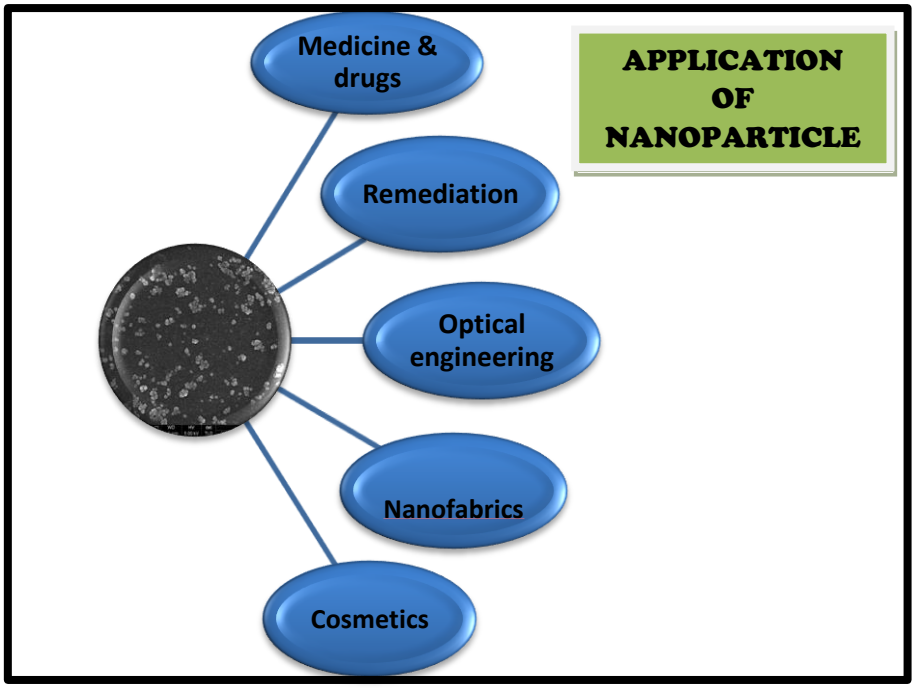

Figure 1: Image depicting several applications of nanoparticles.

Several physical and chemical methods are employed for synthesizing and stabilizing silver nanoparticles[5-7].Chemical approaches are extensively used by virtue of its convenience and rapid synthesis rate. It includes chemical reduction by using various organic and inorganic reducing agents, physicochemical reduction, electrochemical techniques, and radiolysis. There is an increasing demand for green synthesis which involves environmental friendly methods to synthesize nanoparticles. Green synthesis approaches mainly include mixed-valence polyoxometalates, biological, polysaccharides and irradiation method which has certain advantages over conventional methods that involve chemical agents which are found to be associated with environmental toxicity [7].

Nanoparticles of different size and shapes[8, 9], can be synthesized in different material compositions and surface modifications[10]. The synthesized nanoparticles are found to exhibit size[11] or shape dependent properties[12]. There are various synthesis procedures for synthesizing of nanoparticles. The focus of nanoscience is now mainly shifting towards the assembly of individual NPs of high-order structures and nanomaterials. It has prospects in

sensors, [12, 13]supercapacitors[14], drug carriers[15],diodes, photonic and photovoltaic cells, or data storage media application[12].While the analogies between nanoparticulate building blocks at the nanoscale and the atomic building blocks at the molecular scale appears quite appealing for high order structure[16, 17], yet it must be remembered that nanoparticles are rarely monodisperse and no two particles are ever identical unlike atoms. This intrinsic polydispersity of nanoparticles make self-assembly more complex which affects the overall characteristics derived from the size-dependent properties of individual nanoparticles (e.g., magnetic susceptibility surface plasmon resonance, SPR)[12, 18, 19]. In order to synthesize highly ordered structures of nanoparticles with well-defined properties and functions, it is highly desirable to lower down the polydispersity of their nanoparticulate components to achieve maximum stability The ability to modify low-polydispersity particles are important for different applications ,like catalysis where the catalytic activity of the nanoparticles is considered important[20, 21].In several cases like reverse micelles, polymeric stabilizers[22], or thermal decomposition methods , low degree of polydispersity can be attained during particle synthesis. However, the particles need to be purified after the synthesis of nanoparticles[12].Size exclusion based chromatography technique can be employed to extract nanoparticles using SDS as a surfactant in a mobile phase.

In the present scenario, there is a growing interest in biological reduction of metal ions into metal nanoparticles particularly in the field of biology and medicine because of their distinct particle size and shape dependent properties[23, 24]. Bionanotechnology, a new approach, has emerged as the integration between biotechnology and nanotechnology for developing biosynthetic and environmental-friendly technology for the synthesis of nanomaterials. Elemental silver is considered to be very effective antimicrobial agent .Studies have revealed their potential to kill the pathogens and thus they are used as disinfectants in Public Health Care from time immemorial even before the advent of penicillin [25]. Currently, silver

nanoparticles , because of their anti-microbial property, is gaining popularity in medical applications [26]. Silver has been not only proven as an effective tool for retarding and preventing the bacterial infections but also they are found to exhibit wound healing activity. In addition to this , silver is known to exhibit an oligodynamic effect because of its ability to exert bactericidal activity at minute concentrations[27]

The bioreduction of $AgNO_3$ using *Azadirachta indica* (Neem) leaves plant extract is implemented in this study. *Azadirachta indica* has various phytochemicals identified to be carbohydrates, alkaloids, steroids, phenols, saponins and flavonoids [26]. The bioreduction of silver nanoparticle from *Azadirachta indica* is a green synthesis method that exhibit good antibacterial activity [28]. The advantage of using Neem leaves for bioreduction of metal ions is that it is easily available throughout the year. Studies have suggested that biomolecules like protein, phenols, flavonoids and some phytochemicals has ability to reduce the metallic ions(silver, zinc) to the nanosize and also play an important role in the capping of the nanoparticles for its stability[29].

The present study aims at bioreduction of $AgNO_3$ using Neem plant extract,characterization of the nanoparticles ,further purification of nanoparticles using size exclusion chromatography (SEC technique).

LITERATURE REVIEW

Green synthesis provides advancement over chemical and physical method as it is proven to be very cost effective, and also environment friendly, that can be easily scaled up for large scale synthesis .In this method use of high pressure, temperature, energy and toxic chemicals are not required . Silver ,from time immemorial is recognized to have strong antimicrobial effect on microbes [30]. The most important application of silver and silver nanoparticles in medical industry is as topical ointments to prevent infection against burn and open wounds[31].It is also used as a drug delivering agent In this paper we report the synthesis of silver nanoparticles from Neem plant extract , reducing the silver ions present in the solution using plant extract of Neem leaves.

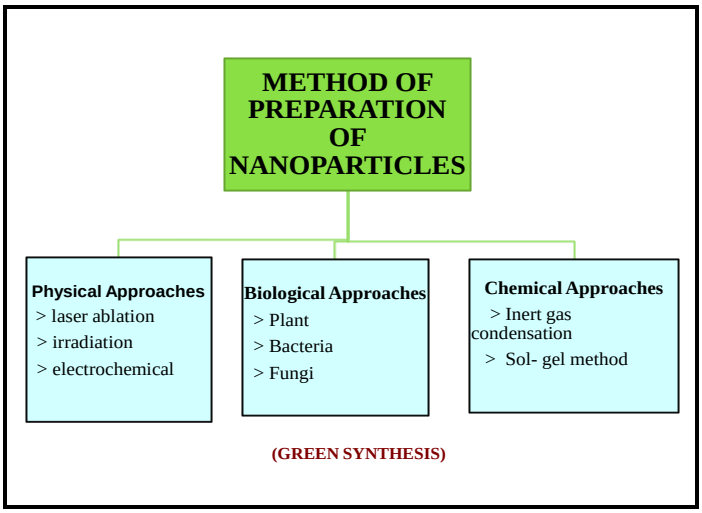

Figure2: Various approaches for the synthesis of nanoparticles .

SYNTHESIS ROUTES OF NANOPARTICLES

Silver nanocrystalline particles can be synthesized by various physical, chemical and biological methods and each synthesized particles show different characteristics in terms of shape and size [**TABLE 1].**From past few years, various chemical methods of synthesizing nanoparticles have been replaced by green synthesis to reduce toxicity while synthesizing nanoparticles and to increase quality and stability [32].

Table 1: Characteristics of synthetic routes for synthesizing silver nanoparticles [33]

METHODS	PRECURSORS	REDUCING AGENT	STABILIZER	PARTICLE SIZE
CHEMICAL	AgNO3	TRISODIUM CITRATE	TRISODIUM CITRATE	NANOSPHERES 30-60 nm
	AgNO3	NaBH4	DODECENOIC ACID	NANOSPHERES 7 nm
	AgNO3	THERMAL DECOMPOSITION	SODIUM OLEATE	NANOSILVER POWDER
PHYSICAL	AgNO3	ELECTRIC DISCHARGE	Sodium citrate	Nanospheres 14-27 nm
	AgNO3	TX-100, UV	TX-100	Nanospheres 30 nm
	Ag wires	Electric Discharge arc	Sodium citrate	Nanospheres 10 nm
BIOLOGICAL SYNTHESIS	AgNO3	*Bacillus*	*Bacillus sp*	Nanospheres 5-15 nm
	AgNO3	*Lactobacillus*	*Lactobacillus sp*	Nanopsheres 6-15 nm
	AgNO3	*Fungus T.viride*	*T.viride*	Nanospheres rod 5-40 nm

APPROACHES FOR NANOPARTICLE SYNTHESIS

Basically there are two main approaches for nanoparticle synthesis i.e. the Bottom up approach and the Top down approach.

Top down approach

In the Top down approach, production of nanosilver involves mechanical grinding of a bulk piece of the material into nanocrystalline particles **(Figure.3).**

Bottom up approach

The Bottom up approach basically involves chemical and biological methods to synthesize nanoparticles. This process requires controlled growth/condensation of solute molecules formed during a chemical reaction. Desired shape and size of the nanoparticles can be achieved by the controlled condensation of nanoparticles. Biosynthesis of nanoparticles is

either scale down or bottom up approach where the main reaction occurring is reduction/oxidation. The microbial enzymes or the phyto-chemicals with antioxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles **[Figure 4][32].**

Figure.3: The top-down approach versus the bottom-up approach.

Figure 4: Plant mediated synthesis of nanosilver.

Physical method of nanoparticles synthesis

Some of the physical methods known for the synthesis of nanoparticles include radiolysis, microwave, ultrasonication, laser abalation and electrochemical methods [32].Metal nanoparticles can be synthesized by evaporation–condensation mechanism , which could be carried out using a tube furnace at atmospheric pressure[34, 35].However, generating silver nanoparticles using a tube furnace has many drawbacks. Tube furnace besides occupying a large space, also consumes a great deal of energy simultaneously raising the environmental temperature around the source material.It is also very time consuming. A typical tube furnace

needs power consumption of more than several kilowatts and a preheating time of several tens of minutes to attain a stable operating temperature[36].

Chemical methods of synthesis of nanoparticles

It is seen that physical methods have limited success and therefore chemical methods for the synthesis of inorganic nanoparticles are widely used [32]. Nanosilver can be synthesized chemically by several processes such as, reduction or oxidation of metal ions, inert gas condensation, or by the sol gel methods[32]. The chemical synthesis process of the silver nanoparticles in solution has the following three main components: (i) metal precursors, (ii) reducing agents (iii) and stabilizing/capping agents. It is also shown that the size and the shape of synthesized Ag-NPs are strongly dependent on these stages. Furthermore, for the synthesis of small sized monodispered silver nanoparticles in a solution, it is highly essential that all nuclei are formed at the same time. In this case, all the nuclei are very much likely to have exactly same or similar size.It will also have same subsequent growth. The initial nucleation followed by the subsequent growth of initial nuclei can be controlled by regulating the reaction parameters such as reaction temperature, pH, precursors, reduction agents (i.e. $NaBH_4$, ethylene glycol, glucose) and stabilizing agents (i.e.PVA, PVP, sodium oleate) [37-39].

Disadvantages of using physical and chemical methods

The synthesis of nanoparticles via physical and chemical processes is very much cost effective. This method requires strong and weak chemical reducing agents as well as capping agents like sodium borohydride, sodium citrate and alcohols. These agents are generally highly toxic, flammable, cannot be easily disposed of.Hence green synthesis of nanoparticles

are now commonly preferred using microorganisms and plant extracts as they are relatively economical, nontoxic and ecofriendly.

GREEN SYNTHESIS OF NANOPARTICLE

Green plant and microorganisms have a remarkable ability to form nanosilver.Biological synthesis using plant materials and microorganisms is proven to be eco-friendly and economical. These days , a large number of microorganisms and plant extracts are used for nanosilver synthesis[32].**Green synthesis is considered as the most ideal synthesis route nowadays** .Extracts obtained from bio-organisms may act as reducing and capping agents in silver nanoparticles synthesis. The reduction of Ag+ ions by means of biomolecules found in these bio-organisms extracts such as amino acids, enzymes/proteins polysaccharides, and vitamins[40]is environmentally benign, yet shown to be chemically complex. An extensive study of literature reports successful silver nanoparticles synthesis using bio-organic compounds. For instance,plant extracts from alfalfa, the broths of lemongrass, geranium leaves etc. have served as green reducing agents in silver nanoparticles synthesis**[Table 2]**. Several microorganisms and microorganisms extract have been also utilized to grow silver nanoparticles intracellularly or extracellularly[40]

Table 2.List of plant synthesizing nanoparticles .[32]

Plant	Size of the particle (nm)
Eucalyptus hybrid (Safeda)	50-150
Azadirachta indica	5-100
Emblica officinalis	10-20
Aloe vera	15±4.2

NEED FOR GREEN SYNTHESIS

The most widely followed method for the biosynthesis of nanoparticles is a type of bottom up approach where the mechanism of reaction is predominantly reduction/oxidation. Green biosynthesis of nanoparticles need was felt as the physical and chemical processes were very expensive[41]. Often, Chemical synthesis method is associated with the environmental toxicity [41]. This issue doesn't arise when it comes to bioreduction of metal ions via green synthesis route[42] .To sum up ,green synthesis provides advancement over all other chemical and physical method because it is proven to be very cost effective, eco-friendly, biocompatible, easily scaled up for large scale synthesis. Other requirements such energy, temperature ,high pressure and toxic chemicals are not present[43]. Hence due the afore mentioned reasons there is a need of silver nanoparticles synthesized by biological methods of plant extract instead of other toxic methods [44].

NANOSILVER:

The extremely small size of nanoparticles is quite significant, because of their large surface area relative to their volume. Particularly silver, have caught the attention of scientists because of its widespread application in the development of new technologies such as electronics, material sciences and medicine [45]. New applications of nanoparticles and nanomaterials are coming up rapidly. Nanocrystalline silver particles have found tremendous applications in the field of high sensitivity biomolecular detection, diagnostics[46],Catalysis ,antimicrobials , therapeutics, and microelectronics[44].

APPLICATIONS OF NANOPARTICLES

Silver Nanoparticle Applications

Silver nanoparticles are not only being used in various technologies but also incorporated into a wide group of consumer products that gets benefits from their unique properties (optical, conductive, and antibacterial properties).

Diagnostic Applications: Silver nanoparticles can be used in numerous assays and biosensors where the silver nanoparticle materials can play a role as biological markers for quantitative detection.

Antibacterial Applications: Their antibacterial property can be incorporated in apparel, wound dressings, footwear, paints appliances, cosmetics, and plastics.

Conductive Applications: Silver nanoparticles are also used in conductive inks and integrated into composites to increase thermal and electrical conductivity.

Optical Applications: Silver nanoparticles can be used to efficiently harvest light and for enhanced optical spectroscopies including metal-enhanced fluorescence (MEF) and surface-enhanced Raman scattering (SERS).

MATERIALS AND METHODS

Preparation of leaf extracts from *Azadirachta indica*(Neem) leaves:

The AR grade silver nitrate ($AgNO_3$) from Sigma-Aldrich was used in the experiment. Fresh leaves of ***Azadirachta indica***, **[Figure5]** was collected from the campus of NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA, ODISHA,INDIA. Leaves were thoroughly washed to remove the dust particles. It was dried in the sun to remove moisture and then grinded to fine powder.1% of Neem leaves was added to 250 ml conical flask containing deionised water followed by stirring for 15 mins. It was incubated for 30 min at 25^o C. Solution was centrifuge at 5000 rpm for 30 min at 25^o C .Clear solution was collected followed by filtration (using 2.5 microns filter paper) with the help of vaccum pump. This solution was used for biosynthesis of silver nanoparticles or reducing the silver ions to metal silver forms

Figure5: ***Azadirachta indica*****leaves.**

Synthesis of silver nanoparticles:

Five different conc.ratios of Plant extract: $AgNO_3$ were prepared i.e. 3.3:1, 30:1, 60:1, 120:1,240:1 by varying the plant extract concentration and keeping the conc.of silver nitrate constant. 1 mM silver nitrate was added to plant extract to prepare a final volume of 200 mL of a solution.Typically, 10 mL of plant extract was added to 190 mL of 1 mM aqueous

$AgNO_3$.A change in the colour of solution was observed during the whole process while taking OD of the solution. The silver nanoparticles solution that was formed was centrifuged at 12,000 rpm for 15-20 min followed by dilution of pellet.

UV-Vis spectral analysis

This bioreduced aqueous component was taken to measure absorbance of the solution. U.V spectra of the following ratio 3.3:1:1, 30:1, 60:1,120:1and 240:1 was taken, out of which 3.3:1 showed the best results. 3.3 ratio OD was noted as time dependent metal ion reduction for 5 hour at an interval of 1 hour between 300 to 500 nm. After 18 hours further optical determination was carried out.

DLS & Zeta-Potential Analysis:

Dynamic light scattering (DLS) which is based on the laser diffraction method was employed to study the average particle size of silver nanoparticles. The prepared sample was diluted in deionised water followed by sonication. Then solution was filtered and centrifuged for 15 min at 25^{o}C at 5000 rpm and the supernatant was separated. The pellet was diluted 4 - 5 times and then the particle distribution in liquid was studied in a computer controlled particle size analyzer (ZETA sizer Nanoseries,Malvern instrument NanoZs).

FTIR spectral analysis

The bioreduced silver nitrate solution was centrifuged at 12,000 rpm for 20 min and the pellet was collected and characterized using ATR-FTIR (Bruker,Germany) in the range of 4000-400cm^{-1}

SEM analysis of silver nanoparticles

Morphology and size of the silver nanoparticles were investigated by SEM images After 24 hours of the synthesis of AgNPs. One drop of sample was put on the glass slide and kept for drying in incubator. Before analysis the samples was coated with gold for 3 min to make it conductive. A thin layer coat of gold was applied to make the samples conductive. Then the sample was characterized by FE-SEM at an accelerating voltage of 5 KV.

X-ray diffraction studies

The solid behaviour of synthesized silver nanoparticles was checked by X-ray diffraction (XRD) spectroscopy .The lyophilized silver nanoparticles powder was characterized by XRD spectroscopy (Philips PAN analytical)using of CU-Kα radiation at a voltage of 30 Kv at a scan rate of 0.05°/min over 2 Θ range of 20-80. The particle size of the prepared samples was determined by using Scherrer′s equation as follows

$$D \approx 0.9\lambda/\beta\cos\theta$$

Where D is crystal size, λ is the wavelength of X-ray, Θ is the Braggs angle and B is the full width at half maximum of the peak in radians.

Purification of synthesized silver nanoparticles using size exclusion chromatograpy

The bioreduced silver ion was centrifuged at 12000 rpm followed by sonication for 10-20 mins. The above solution was diluted with deionised water .The contents were filtered using 0.22 microns filter paper. O.D was taken of samples diluted several times .Solution was

filtered using 0.22 micron filter paper to separate out the nanosized materials from the bigger molecules For purification of silver nanoparticles ,size exclusion chromatography was employed. It consists of mobile phase and stationary phase. The principle of chromatography is based on the differences in size of the particles. Small sized particles moves slowly through the columns while the bigger particles which do not fit into the pores are eluted out first from the void volumes. In our experiment , mobile phase was a mixture of SDS(10mM) and deionised water, stationary phase constituted Sephadex G100 ,bead size 40-120 micrometer with a pore size cut off of 100 KDs. SDS is added to the mobile phase to prevent adsorption of nanoparticle onto the stationary phase, and also to dissolve moieties present on the surface of the nanoparticles. The injected solution volume was 10mL.Sample was loaded onto the column and eluents were collected at a flow rate of 1mL/min.To analyze the size and dispersity of particles,different eluents collected on the basis of their retention volume and time were characterized using UV-VIS spectra, DLS and Zeta potential. Samples were lyophilized and stored for future use.

RESULTS AND DISCUSSION

UV-Vis spectra analysis after synthesis of silver nanoparticles

Bioreduction of silver ions into AgNP after exposure to Neem plant extract was observed.A distinct colour change in the solution was seen .The colour of the solution changed from pale yellow to brown as shown in **figure 6**.The sharp clear intense bands of silver nanoparticles were observed at 430 nm in case of *3.3: 1 ratio* ,whereas in the ratio 30:1 a broad peak was observed , while in 60:1 ,120 :1 and 240:1 ratio, peak was observed at 379nm,388nm and 399nm respectively **[Figure 7]**. From several literatures it was reported that the silver nanoparticles usually show SPR peak centred at 420 nm. From our experiments, we found the SPR peak for silver nanomaterials at 430 nm in 3.3:1**[Figure 7]**. So, Taking this optimum ratio(3.3:1), further work on synthesis and extraction of silver nanoparticles was carried out. A steady increase in the intensity of the absorption peak was observed for OD taken in 4 hours, 5 hours and 18 hours for the ratio 3.3:1**[Figure 8].** Initially the solution colour was light yellowish than with time duration it turned from yellowish to light brown to brownish, after this no further colour change was observed

Colour change indicates the formation of silver nanoparticles ,This formation demonstrates that that silver ions in aqueous medium have been converted to nanosized elemental silver. It is known very well that silver nanoparticles have a distinguished brown color in aqueous solution because of the surface plasmon resonance in silver nanoparticles. The metal (silver) nanoparticles have free electrons, which are responsible for the SPR absorption band. Colour change was observed till 18 hours This was the point where almost all the metal ions were converted into nanoforms. The shift of the bands in the other ratios were probably due to the presence of air bubbles at interface whereas in time dependent bioreduction reaction the peak wavelength did not shift during the whole reaction ,suggesting that size of nanoparticle remains unchanged thorough the whole reaction.Hence, we can conclude that low conc. of

plant extract is optimum to synthesize nanoparticles The silver nanoparticles were observed to be stable in solution even after several weeks of their synthesis.

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Figure 6: Left, 1mM AgNO3 without plant extracts.Right,1mM AgNO3 with Neem extract after 18 hours .

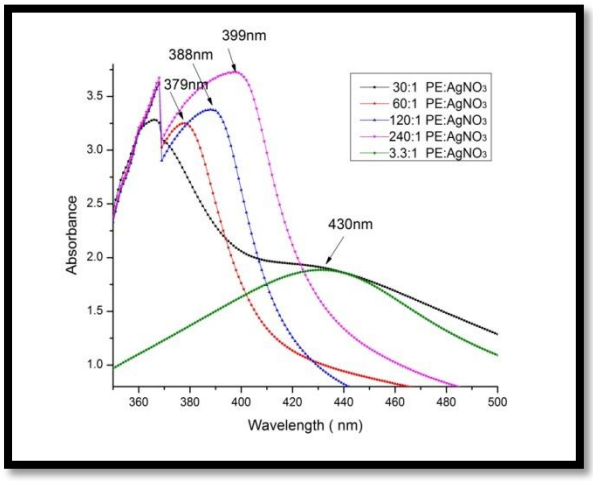

Figure.7

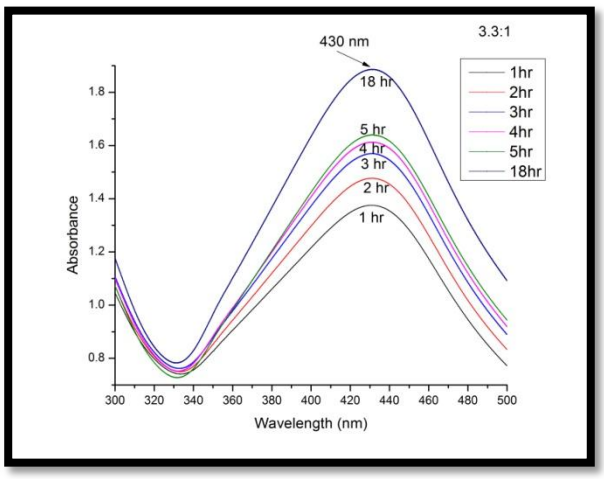

Figure.8

Figure7: Shows the absorption spectrum biosynthesized silver nanoparticles from Neem plant extract of 5 ratios, 30:1,60:1,120:1,240:1 and 3.3:1.

Figure 8: UV- visible spectra of *Azadirachta indica*as a function of time in different incubations with silver nitrate (1mM) after 4h, 5h and 18 h respectively. The peak 430nm corresponds to the plasmon resonance of silver nanoparticles.

DLS ANALYSIS

Figure 9 shows the particle size distribution of silver nanoparticles of 3.3:1 ratio. The average size of the nanoparticles is 104.8

The size of the nanoparticle is appropriate as it is around 100 nm. The size of the synthesized particles is slightly bigger than usual range of nanoparticles size 1-100 nm perhaps because of plant proteins adsorbed on the surface of the nanoparticle, which confers stability by preventing them from agglomerating.

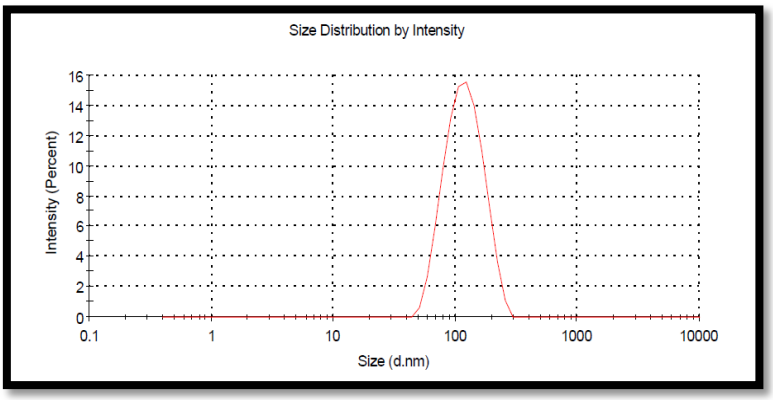

Figure 9: DLS result for 3.3:1 ratio silver nanoparticles.

ZETA ANALYSIS

Zeta potential detected for silver nanoparticle was -20.8 mV **[Figure10].** Nanoparticles having charge between from ±10 to ±30 are quite stable

Zeta potential (surface potential) has direct relation with the stability of a nanoparticles formed as mentioned in **Table 3**. Since Nanoparticles are in nano forms they have a tendency to agglomerate /aggregate to stabilize themselves, as they are energetically very unstable. Therefore particles undergo agglomeration/aggregation to stabilize themselves. So, potential charges on the surface of the nanoparticles make them stable and prevent from getting aggregated.

Table 3: Stability of the NPs vs. surface potential (Zeta potential).

Zeta potential [mV]	Stability behaviour of the colloid
from 0 to ±5	Rapid coagulation or flocculation
from ±10 to ±30	Incipient instability
from ±30 to ±40	Moderate stability
from ±40 to ±60	Good stability
more than ±61	Excellent stability

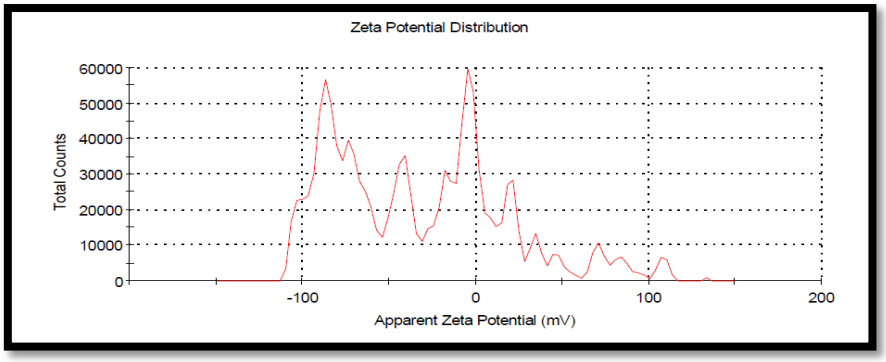

Figure.10: Zeta analysis result for 3.3:1 ratio silver nanoparticles.

FE SEM ANALYSIS:

FE SEM analysis provided further detailed insight into the morphology and size details of the silver nanoparticles. Our experiments results showed that the size of synthesized nanoparticles in the colloidal solution ranges from 50-83 nm [**Figure11].** Particles observed are predominantly spherical in shape and they are quite well distributed without any agglomeration.

The synthesized nanoparticles were well stabilized by capping agent (plant phytochemicals) hence they were not in direct contact even within the aggregates as seen in SEM image Since these phytochemicals are involved in bonding with nanoparticles they provide charge to the nanoparticle .Repulsion due to the same charges between the particles keep them from getting clumped together .this is further confirmed by Zeta and FTIR analysis.

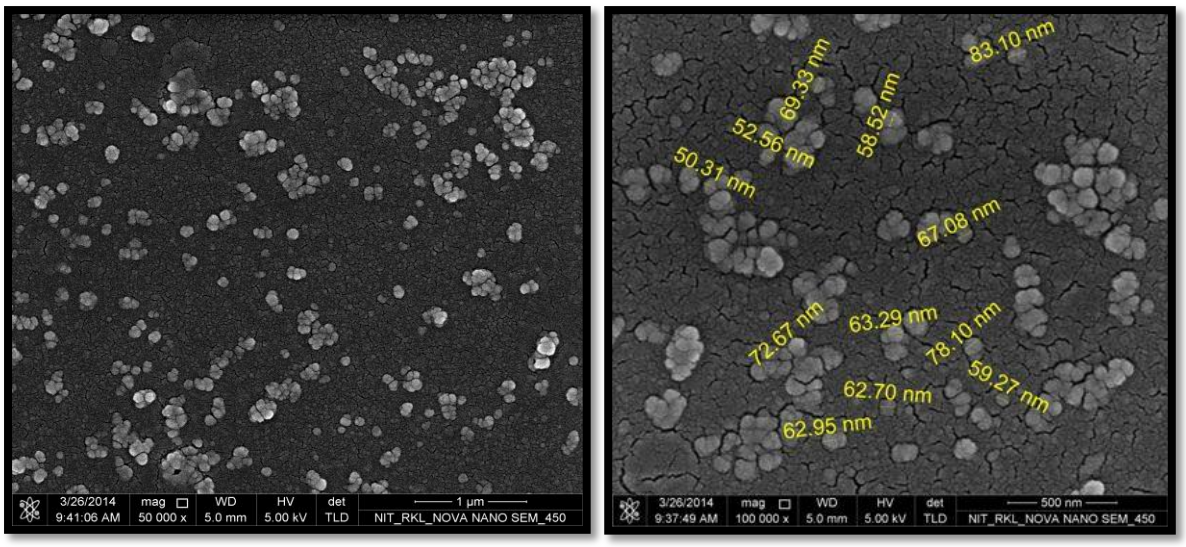

Figure 11: SEM image for 3.3:1 ratio silver nanoparticles.Particles are small sized(1-100nm)spherical and well distributed

XRD ANALYSIS:

XRD data shows the shape and nature of the synthesized material. It confirmed that the synthesized silver nanoparticles are of crystalline nature. Intense Bragg reflections can be due to presence of nanoparticle with respective miller indices **[Figure12].**XRD analysis showed distinct diffraction peak indexed as 110.These sharp narrow Bragg peaks proves that the synthesized silver nanoparticles are of crystalline nature and FCC type of closely packed structure. Intense Bragg reflections can be due to capping agents that result due to strong X-ray scattering centres in the formed nanocrystals.

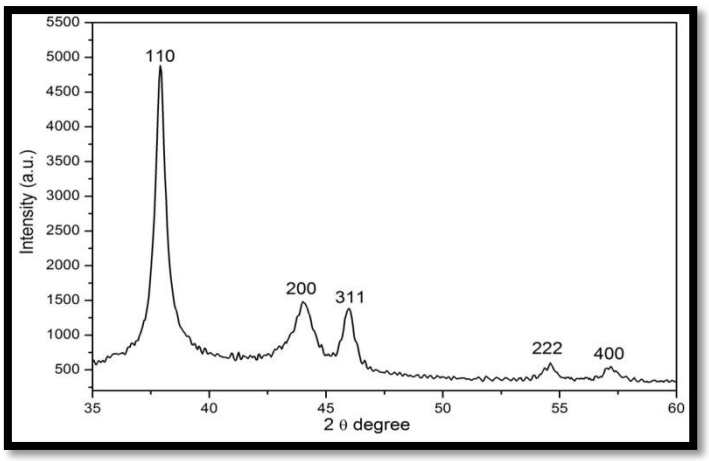

Figure 12: XRD result for 3.3:1 ratio silver nanoparticles.

FTIR ANALYSIS:

FTIR spectrum was analysed for identification of different biomolecules adsorbed on the surface of nanoparticles, and also to find out their role in reduction and stabilizing the nanoparticles The FTIR spectrum of synthesized silver nanoparticles by the *Azadirachta indica* leave extract, **figure 13**shows strong bands at 3550,2362,1648,1540,1510,1458 and 518 cm^{-1}. 3550 cm^{-1} corresponds to O-H groups, H bonded alcohols and phenols.A peak at 1648 cm^{-1} corresponds to secondary amine. The spectral bands (1450-1600 cm^{-1}) show presence of proteins which are responsible for the reduction of metal ions or affinity for metal nanoparticles.The bands (1300-1450 cm^{-1}) suggest the presence of flavanones/terpenoids adsorbed on the surface which are very abundant in *Neem* plant,while nanoparticles bond showed strong peak at518 cm^{-1}.

From the analysis of the FTIR spectrum, carboxyl group were found adsorbed on the particles surface, hence that confirms the presence of biomolecules like terpenoids,flavonoids which acts as a capping agent for the synthesized nanoparticles.This also throw some light on the dual role of biological molecule in reducing metal ions and capping .Capping of nanoparticles by protein stabilizes silver nanoparticles and prevents agglomeration in the medium. FTIR

analysis confirmed that the *Azadirachta indica* leaves extract can perform dual functions of reduction of (Ag^{+}) to (Ag^{o}) and also stabilization of silver nanoparticles.

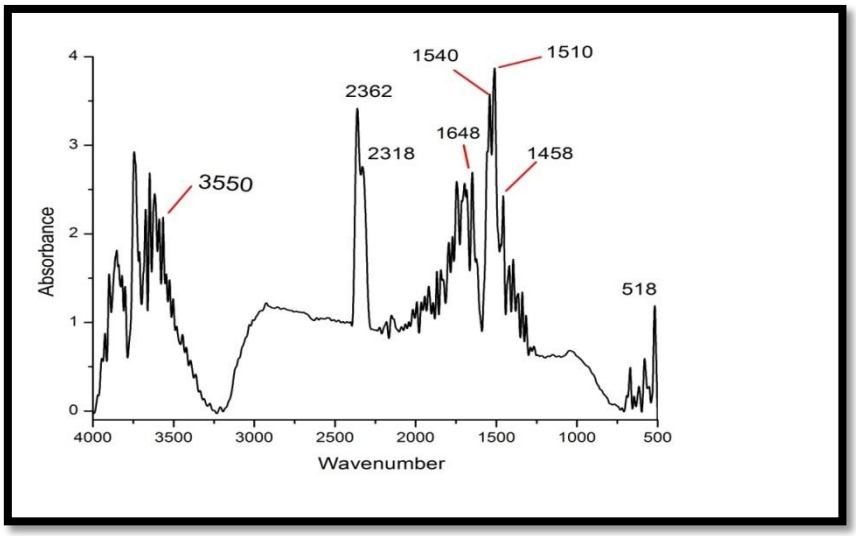

Figure 13: FTIR result for 3.3:1 ratio silver nanoparticles.

PURIFICATION AND ISOLATION OF SYNTHESIZED NANOPARTICLES

Purification of nanoparticles was characterized using UV-Vis spectra and DLS.Silver nanoparticles of varying sizes were separated using mobile phase addictive(10mM SDS) at a constant flow rate of 1.0mL/min [**Figure 14].** Accordingly,it was seen that different sized nanoparticles eluted according to the retention time. In order to validate the reproducibility of elution relatively standard deviation of the elution times from 4 consecutive runs were analyzed.

Figure 14: Purification of nanoparticles using size exclusion chromatography

UV Spectra analysis after purification of synthesized nanoparticles

Figure15,shows that the intensity of absorption peak sharply increases in eluent 1 ,2,3 and then decreases in eluent 4 while in eluent 5, 6, 7,8 absorbance is almost parallel to that of absorption of control. But some peak have been detected in range 200-280 nm in all eluents

Sharp intensity increase is due to increase conc. of nanoparticles in eluent. More the conc. of nanoparticle in eluent ,more the SPR,Highest peak in eluent 4 corresponds to the highest conc. of nanoparticles No absorption peak is detected in 5,.6,7,8which suggests that nanoparticles conc. is almost nil in that eluted fraction hence no SPR peak. Peaks in the range 200-280 nm indicate that certain protein are tightly bounded to the nanoparticles that shows there absorbance at characteristic wavelength Absorbance in the afore mentioned regions in 5-8 eluents indicates the presence of extremely small sized protein that were present in the solution.

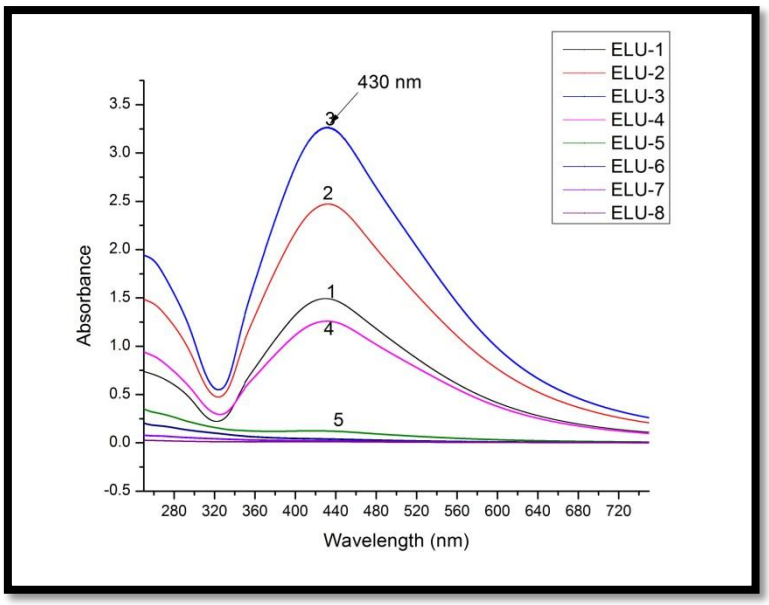

Figure 15:UV visible spectra of different Eluents of 3.3:1 ratio silver nanoparticles.

DLSand ZETA analysis of purified silver nanoparticles

SEC gave fractions in which average nanoparticles size decreased with elution time**.** It was confirmed from the DLS analysis that comparatively larger sized nanoparticles were eluted first from the accessible volume or the void volume of the column.This was followed by the smaller particles that meanders freely and travel steadily down the column from the pores according to their retention time.Comparative analysis of the average size and charge of eluent 1,2,3 and 4 were as follows **Table 4.**

On analysis nanoparticles of size 93.88 were found to be100% pure while nanoparticles in eluent1 3 and 4 were 98% pure.It might be due to the presence of some proteins moieties on their surface that were present as corona and hence size are comparatively bigger than ELU 2.

Table 4:Comparative analysis of Eluent's size and zeta potential.

ELUENT	DLS AVERAGE SIZE (d.nm)	DEVIATION (d.nm)	%INTENSITY	ZETA POTENTIAL(mV)
Eluent 1 Figure .16	99.54	49.18	98.8	-23.8
Eluent 2 Figure.18	93.88	33.07	100	-33.0
Eluent 3 Figure.19	97.04	44.57	98	-44.2
Eluent 4 Figure.20	96.11	44.57	97.5	-43.4

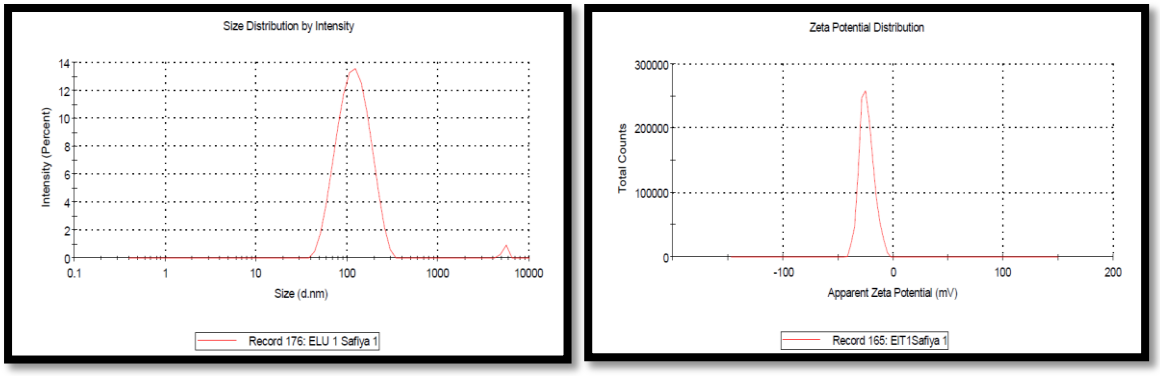

Figure16: Image showing DLS of eluent 1'saverage size is 99.54 d.nm and Zeta is -23.08 mV.

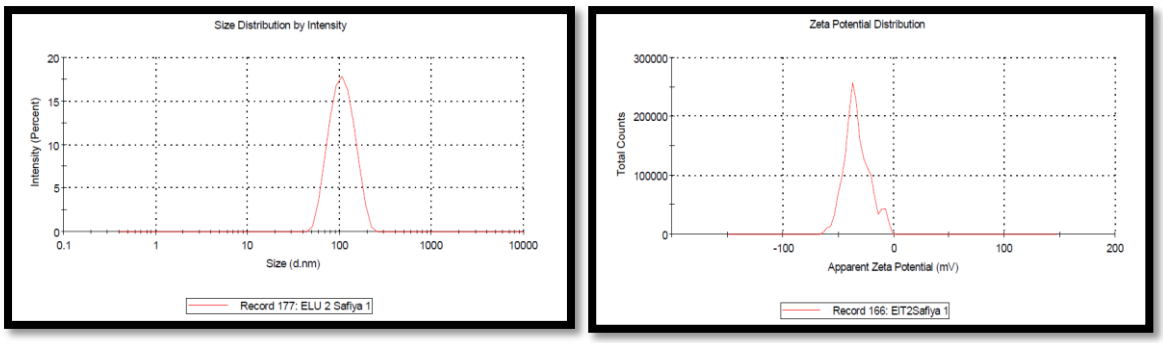

Figure 17:Image showing DLS of eluent 2's average size 93.88d.nm and ZETA -33.0mV

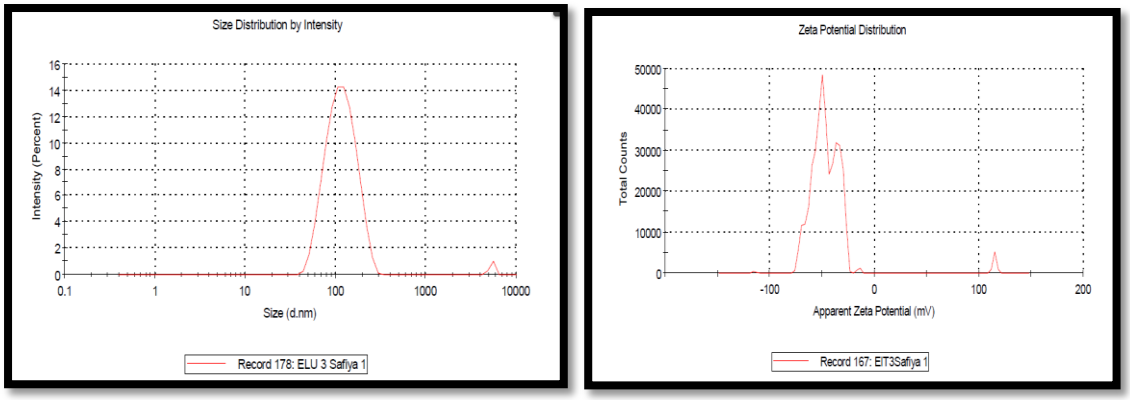

Figure18: Image showing DLS of eluent 3'saverage size is 97.04 d.nm andZeta is-44.2mV.

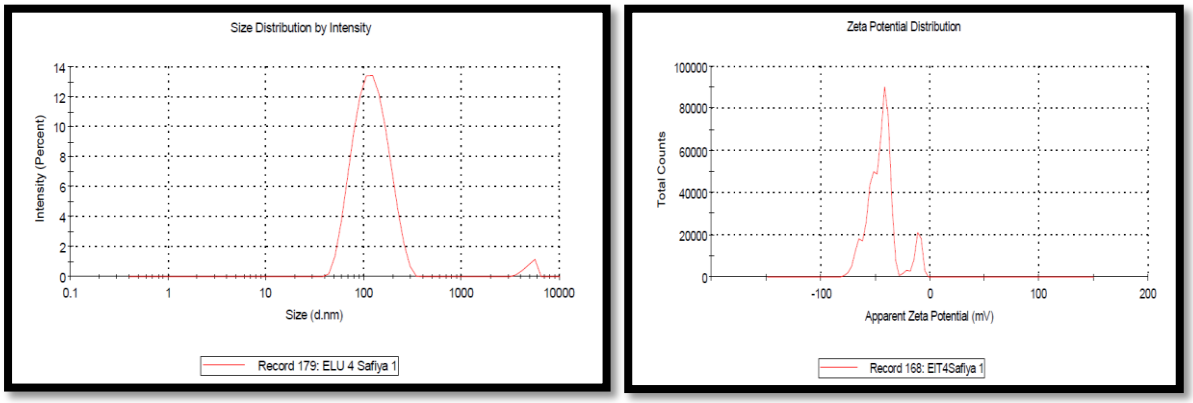

Figure 19:Image showing DLS of eluent 4'saverage size is 96.11 d.nm.and Zeta is -43.4mV.

CONCLUSION

The present works represents an economical, non-toxic and eco-friendly method for synthesizing silver nanoparticles. These silver nanoparticles showed characteristic absorption peak at 430 nm in UV spectra.It is clearly seen that the *Azadirachta indica* leaf extract successfully reduce silver ions to silver in nanoforms in the ratio of 3.3:1 *Azadirachta indica* leaf extract and silver nitrate.Further characterization of synthesized nanoparticles using SEM showed the nanoparticles are small sized (1-100nm), spherical in shape and well distributed. Charge on the synthesized nanoparticles is in the range of -20.8 mV. Lower ratios of plant extract are optimum for synthesizing small sized nanoparticles within the range 1-100 nm.The characterization of purified nanoparticles after size exclusion chromatography using DLS and Zeta, showed the nanoparticles size and charge in the range of 90-99 d.nm and 20-50 mV respectively, which indicates that SDS plays a key role in reducing the size and stabilizing the nanoparticles. The role of terpenoids and flavanoids as a bioreductant and capping agent carrying functional group like aldehyde, amine, ketones was further illustrated by FTIR.

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